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UV-induced DNA-binding proteins in human cells

(SOS response/HeLa cells)

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ABSTRACT **To investigate the response of human cells to DNA-damaging agents such as UV irradiation, we examined nuclear protein extracts of UV-irradiated HeLa cells for the presence of DNA-binding proteins. Electrophoretically separated proteins were transferred to a nitrocellulose filter that was subsequently immersed in a binding solution containing radioactively labeled DNA probes. Several DNA-binding proteins were induced in HeLa cells after UV irradiation. These included proteins that bind predominantly double-stranded DNA and proteins that bind both double-stranded and single-stranded DNA. The binding proteins were induced in a dose-dependent manner by UV light. Following a dose of 12 J/m², the binding proteins in the nuclear extracts increased over time to a peak in the range of 18 hr after irradiation. Experiments with metabolic inhibitors (cycloheximide and actinomycin D) revealed that *de novo* synthesis of these proteins is not required for induction of the binding activities, suggesting that the induction is mediated by protein modification.**

Throughout nature, cells are continually exposed to agents that damage their genetic material. Studies with *Escherichia coli* have revealed specific, inducible pathways by which the bacterial cells respond to DNA-damaging agents (reviewed in ref. 1). These pathways involve cellular functions by which *E. coli* repair and replicate damaged DNA, sometimes in an error-prone fashion. There is accumulating evidence that mammalian cells also have inducible pathways by which they respond to DNA-damaging agents. Early work in our laboratory (2, 3) suggested that there is an inducible, SOS-like response in mammalian cells exposed to DNA-damaging agents such as UV light. It was shown that the UV reactivation of herpes simplex virus is mutagenic and inducible in mammalian cells (2), in a manner analogous to Weigle reactivation of bacteriophage (4). It was further demonstrated that error-prone mutagenesis can be detected in mammalian cells pretreated with DNA-damaging agents, but using a plasmid shuttle vector as a probe for mutagenesis in monkey COS cells (3). These studies defined several aspects of what some have called the mammalian stress response, but the mechanisms underlying these observations have yet to be elucidated. Miskin and Ben-Ishai (5) determined that plasminogen activator is induced in fibroblasts by UV light, but the significance of this induction is unclear. Work by Rotem *et al.* (6) and by Schorpp *et al.* (7) revealed that at least part of the mammalian stress response can be mediated by an extracellular factor. Angel *et al.* (8) identified mRNA species that accumulate in response to phorbol esters [e.g., phorbol 12-myristate 13-acetate (PMA)] and in response to UV light. Angel and coworkers (9) analyzed the phorbol ester-responsive element in some of these genes, and they and Lee *et al.* (10) found that the cellular protein AP1 binds to this element and may play a role in the response. The genes found to be PMA-inducible, however, such as c-*fos*, c-*myc*, and those encoding metallothionein, collagenase, and stromolysin, do not have any clear role in DNA repair or mutagenesis. We have sought to identify stress-related proteins that may be involved in DNA repair or mutagenesis, and we reasoned that proteins that bind to DNA would be of interest. Using the technique of protein–DNA blotting (11), we have detected several UV-induced DNA-binding proteins in human cells.

MATERIALS AND METHODS

Cells. HeLa S3 cells (obtained from David Ward of Yale University) were grown in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum. Prior to irradiation, cells were distributed to 150-cm² plates at a density of 25,000 cells per cm². Forty-eight hours later, the cells were washed with phosphate-buffered isotonic saline (PBS) and irradiated with 254-nm UV light at a dose of 12 J/m², unless otherwise indicated. Fresh MEM with 10% calf serum was added to the cells immediately following UV treatment. Mock-irradiated cells were simply washed with PBS and placed in fresh medium without irradiation. Eighteen hours later, nuclear proteins were prepared from the cells. Studies using metabolic inhibitors were carried out on irradiated and unirradiated cells. After irradiation or mock irradiation, fresh MEM with 10% calf serum containing either cycloheximide at 25 μg/ml or actinomycin D at 10 μg/ml was added to the cells, and nuclear proteins were prepared 12 hr later. Pulse-labeling experiments to measure protein and RNA syntheses were carried out in parallel using [^{35}S]methionine and [^{3}H]uridine, respectively. Parallel cultures were labeled for 1-hr periods at the beginning, middle, and end of the 12-hr incubation.

Protein Preparation. Nuclear proteins were prepared from the cells exactly as described by Miskimins *et al.* (11). In brief, cells were lysed in 0.5% Triton X-100/10 mM Hepes, pH 8.0/0.5 M sucrose/50 mM NaCl/10 mM $MgCl_2$/1 mM dithiothreitol. Nuclei were collected by centrifugation, and the nuclei were lysed in cell lysis buffer plus 0.5 M NaCl and 10 mM spermidine. High molecular weight DNA was removed from the lysate by centrifugation, and the supernatant was saved and dialyzed against 50% (vol/vol) glycerol/10 mM Hepes, pH 8.0/50 mM NaCl/10 mM $MgCl_2$/1 mM dithiothreitol.

Protein–DNA Blots. As described by Miskimins *et al.* (11), proteins were separated in either 7% or 10% polyacrylamide gels in the presence of SDS without prior boiling of the samples. The proteins were transferred electrophoretically

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

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from the SDS/polyacrylamide gels to nitrocellulose filters. The filters were soaked in a blocking buffer (10 mM Hepes, pH 8.0/5% non-fat dry milk) at room temperature for 1 hr. The filters were then incubated for 1 hr at room temperature in binding buffer (10 mM Hepes, pH 8.0/50 mM NaCl/10 mM $MgCl_2$/1 mM dithiothreitol/0.25% non-fat dry milk) containing a radioactively labeled DNA probe. The filters were washed for 1 hr at room temperature in wash buffer composed of binding buffer without the DNA probe and containing 150 mM NaCl instead of 50 mM NaCl. The filters were then subjected to autoradiography to visualize the DNA-binding proteins.

DNA Probes. In most experiments, wild-type λ phage DNA digested with *Hin*dIII was used as the DNA probe. To generate double-stranded DNA (dsDNA) probes, the restriction fragments were labeled by Klenow polymerase-mediated fill-in of the cohesive ends with [α-^{32}P]dCTP and [α-^{32}P]-dATP. Single-stranded DNA (ssDNA) probes were produced by random primer extension using λ DNA as a template, followed by heat denaturation prior to use in the binding buffer. Radioactively labeled DNA was added to the binding buffer to yield 5×10^5 cpm per ml.

RESULTS

We exposed HeLa cells in the exponential phase of growth to 254-nm UV light (12 J/m^2). Nuclear proteins were prepared 18 hr later from these cells and from unirradiated control cells. The proteins were separated by electrophoresis and were transferred from the gels to nitrocellulose filters electrophoretically. DNA-binding proteins were visualized by autoradiography after incubating the filter-bound proteins in the presence of radioactively labeled DNA probes dissolved in a milk-containing binding buffer. Fig. 1 shows the results of three such analyses of proteins present in UV-irradiated cells. We examined the binding of these proteins to radioactively labeled DNA probes consisting of either double-stranded λ DNA restriction fragments, single-stranded λ DNA restriction fragments, or a mixture of the two. There is induction by UV light of at least five proteins in HeLa cells that bind dsDNA. Two of these induced proteins also bind ssDNA. That the DNA binding is conformation-specific, in that some of the proteins bind only dsDNA, whereas some bind both dsDNA and ssDNA, suggests that the nature of the interactions involved in the binding is more specific and significant than basic proteins attracting acidic nucleic acids. The approximate molecular masses of the dsDNA-binding proteins are 130, 110, and 98 kDa; the proteins that bind both ds- and ssDNA are approximately 93 kDa and 85 kDa. In the absence of any information about their biological function, we have designated these proteins by their apparent sizes and by the fact that they are damage-inducible: DI-130, DI-110, DI-98, DI-93, and DI-85. Since the proteins are not boiled prior to electrophoretic separation in this method, the above estimates of molecular mass may not be exact. The intense signal in the low molecular weight region seems to represent DNA-binding proteins that are only slightly induced by UV light. Based on the observations of Miskimins *et al.* (11), this signal may in part be related to histone proteins. In the 7% polyacrylamide gel used in this experiment, however, this signal was not sufficiently resolved for adequate analysis. In repeated experiments, we noticed that the relative signal intensities from the induced proteins were somewhat variable, and additional induced bands were sometimes detected. However, the factors affecting this variability are not known. The induction of these proteins cannot be visualized by Coomassie blue staining of protein gels; in fact, no differences in the patterns of gel-fractionated proteins from irradiated and mock-irradiated cells could be detected by this staining technique (data not shown).

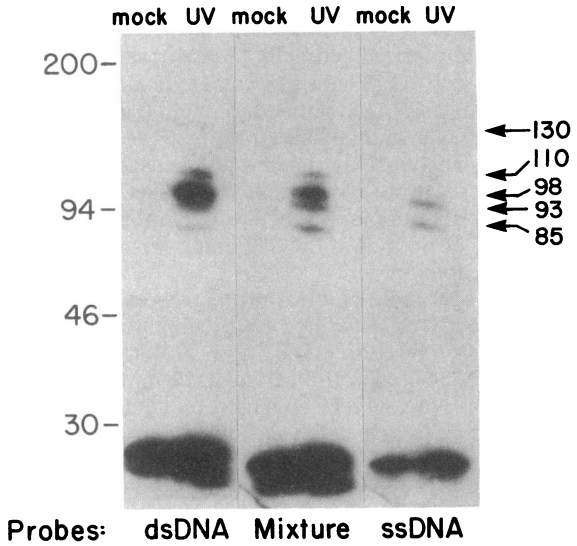

FIG. 1. UV induction of DNA-binding proteins in HeLa cells. Cells were irradiated with 254-nm UV light at 12 J/m^2, and 18 hr later nuclear proteins were prepared from irradiated or mock-irradiated cultures. The proteins were fractionated by electrophoresis in an SDS/7% polyacrylamide gel and were transferred to nitrocellulose filters. The filters were incubated in binding buffer containing radioactively labeled DNA to allow autoradiographic detection of DNA-binding proteins. As indicated, the radioactively labeled DNA consisted of either λ phage dsDNA restriction fragments, λ ssDNA restriction fragments, or a mixture of the two. Locations and sizes (kDa) of marker proteins are indicated at left. Arrows at right indicate five bands that represent UV-induced DNA-binding proteins, with approximate molecular sizes given in kilodaltons.

No obvious sequence specificity of the observed binding has been detected. Similar results (not shown) were obtained with total human genomic DNA, the herpes simplex virus thymidine kinase gene, the mouse metallothionein I gene promoter, and pBR322 DNA as probes in place of λ phage DNA.

Fig. 2 shows that the induction of the DNA-binding activity by UV light is dose-dependent. The activity detected by a double-stranded λ DNA probe increases with increasing dose from 0 to 24 J/m^2, a dose that reduces cell survival as judged by colony-forming-ability to <1% of that seen with unirradiated cells. The clear dose-related response to UV irradiation indicates that this phenomenon is a direct effect of UV irradiation on exponentially growing HeLa cells. In this particular experiment, DI-110 was seen only faintly, whereas two induced bands at 27 and 25 kDa were visualized. This experiment was performed with a 10% polyacrylamide gel instead of a 7% gel as in Fig. 1; consequently, the low molecular weight signal seen in Fig. 1 was resolved into various component bands, all representing proteins of apparent molecular mass <20 kDa. These appear to be DNA-binding proteins that are not significantly affected by UV light.

The time course of the induction of the DNA-binding activity is shown in Fig. 3. The binding activity increases with time after UV treatment of the cells, with the highest activity observed in this experiment at 18 hr, followed by a slight decline by 24 hr. Because only four time points were taken in this experiment, the precise time of peak activity cannot be defined. Also, it is possible that the several induced proteins are induced in slightly different temporal patterns that were not differentiated in this experiment. Nonetheless, the basic trend is clear. This time course is consistent with the hypothesis that the increased binding activity is generated after the cells have sensed the damage produced by the UV radiation. The detection of this damage may be related to the

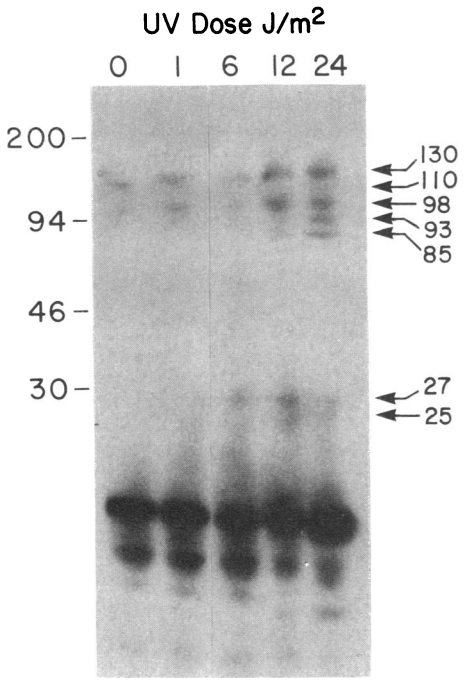

FIG. 2. UV dose dependence of the induction of DNA-binding proteins in HeLa cells. Cells were irradiated with 254-nm UV light as indicated, and 18 hr later nuclear protein extracts were prepared from the cells. The proteins were fractionated by electrophoresis in an SDS/10% polyacrylamide gel and were transferred to nitrocellulose filters. The filters were incubated in binding buffer containing radioactively labeled λ phage dsDNA restriction fragments to allow autoradiographic detection of DNA-binding proteins. Locations and sizes of marker proteins are indicated at left. Arrows at right indicate seven bands that represent UV-induced DNA-binding proteins.

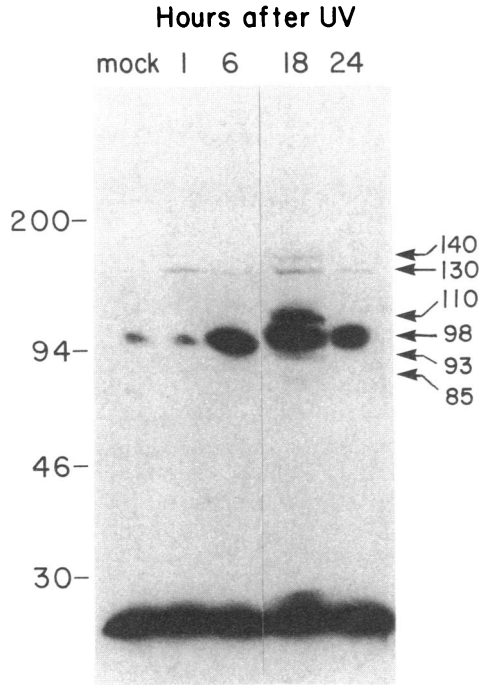

FIG. 3. Time course of the UV induction of DNA-binding proteins in HeLa cells. Cells were irradiated with 254-nm UV light at 12 J/m^2, and at the indicated times after irradiation, nuclear protein extracts were prepared from the cells. Protein extracts were prepared from mock-irradiated cells 18 hr after the mock treatment. The proteins were fractionated by electrophoresis in an SDS/7% polyacrylamide gel and were transferred to nitrocellulose filters. The filters were incubated in binding buffer containing radioactively labeled λ phage dsDNA restriction fragments to allow autoradiographic detection of DNA-binding proteins. Marker proteins are indicated at left. Arrows at right indicate six bands that represent UV-induced DNA-binding proteins.

cell cycle, and so a peak effect occurring about 18 hr after irradiation is consistent with the accumulated response of asynchronized HeLa cells, whose doubling time is normally about 1 day. In this experiment, an additional band at ≈140 kDa (see below) was evident along with the five bands seen in Fig. 1.

The effects of inhibitors of protein and RNA syntheses on the induction of these binding proteins were examined. Immediately after irradiation or mock-irradiation, cells were placed in medium containing sufficient actinomycin D or cycloheximide to reduce RNA and protein syntheses to 4% and 2% of normal, respectively, as judged by pulse-labeling experiments done in parallel. Nuclear proteins were prepared for analysis 12 hr later (Fig. 4). Neither actinomycin D nor cycloheximide had an effect on the induction of the DNA-binding proteins. These results suggest that the UV-induced DNA-binding activity does not come from newly synthesized proteins but arises from preexisting proteins that may become modified and altered in activity in some way, perhaps by phosphorylation or by proteolytic cleavage.

The slight variability in the number of induced bands is again evident in Fig. 4. In this experiment, DI-140 was again present and induced by UV, but DI-110 was seen only faintly. Also, as in Fig. 2, DI-27 and DI-25 were seen to be induced by UV, as was a protein of 35 kDa that was not previously detected. Note also that the very low molecular weight bands seen in the previous figures were run off this particular gel.

Another aspect of variability in these experiments is that we have occasionally found that the DNA-binding proteins are present in mock-irradiated cells. After careful examination of cell growth conditions, we have learned that serum starvation (i.e., growth in 0.5% calf serum for 2 days) can induce the same pattern of DNA-binding proteins as is seen with UV irradiation of the cells (data not shown). This finding suggests that the DNA-binding proteins are not generated exclusively in response to DNA damage. Other stresses placed upon the cells, such as alterations in growth conditions, may stimulate pathways that lead to enhanced DNA-binding activity among nuclear proteins. The fact that growth conditions, such as serum starvation, can induce certain proteins that are otherwise inducible by UV or PMA was also noted by Schorrp *et al.* (7).

In light of the work of Miskin and her colleagues (5, 6) and of Herrlich and his colleagues (7, 8), it is reasonable to ask whether the induction of DNA-binding proteins by UV light can be mediated by an extracellular factor. We transferred medium from UV-treated cells at various times after UV treatment to unirradiated cells. This application of conditioned medium to unirradiated cells failed to induce any DNA-binding activity as measured in our assay (data not shown). However, we did not test for the presence of the factor that Herrlich and coworkers described by any of the assays that they used, and we used HeLa cells whereas they used human primary fibroblasts.

In other experiments, we found that the DNA-binding activity observed was not enhanced by the presence of ATP in the binding buffer (data not shown). In various competition assays, we determined that the induced proteins bound equally well to UV-irradiated and unirradiated DNA (data not shown).

In immunoblots done in parallel with the protein–DNA blots, we found that these DNA-binding proteins are distinct from the Ku protein described by Mimori *et al.* (12) and that the Ku protein in HeLa cells is not further induced by UV light (data not shown). Using antibodies raised against *E. coli* RecA protein, we detected no crossreactivity between RecA

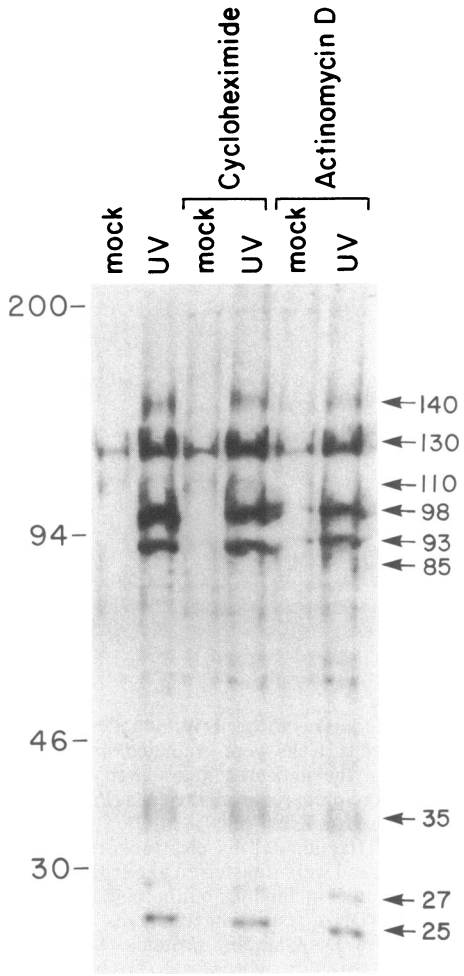

FIG. 4. Failure of cycloheximide or actinomycin D to inhibit the UV induction of DNA-binding proteins in HeLa cells. After mock-irradiation or irradiation with 254-nm UV light at 18 J/m^2, cells were incubated in growth medium containing either no added inhibitor, cycloheximide at 25 μg/ml, or actinomycin D at 10 μg/ml. Twelve hours later, nuclear protein extracts were prepared from the cells. The proteins were fractionated by electrophoresis in a SDS/7% polyacrylamide gel and were transferred to nitrocellulose filters. The filters were incubated in binding buffer containing radioactively labeled double-stranded and single-stranded λ phage DNA restriction fragments to allow autoradiographic detection of DNA-binding proteins. Marker proteins are indicated at left. Arrows at right indicate nine bands that represent UV-induced DNA-binding proteins.

and any of the UV-induced DNA-binding proteins we describe (data not shown).

DISCUSSION

The detection of DNA-binding proteins induced in HeLa cells by UV irradiation may represent a novel aspect of the mammalian SOS response. Such a response is reminiscent of the RecA response in *E. coli* (1), but specific similarities to the SOS response in *E. coli* have not yet been demonstrated. Physiologic roles for these proteins are not known, but it is reasonable to speculate that proteins that are induced by UV irradiation and that bind to DNA without obvious sequence specificity may be involved in DNA metabolism such as repair or mutagenesis.

In several experiments, a total of nine DNA-binding proteins were detected. The results presented in Fig. 1 demonstrate that two of these proteins bind both dsDNA and ssDNA, whereas the other three seen in Fig. 1 bind only dsDNA. In the experiments represented by Figs. 2–4, four additional proteins that bind dsDNA were detected; it was found that these four proteins do not bind ssDNA (data not shown). Hence, of the nine UV-induced DNA binding proteins, two bind both ds- and ssDNA and seven bind only dsDNA. This observation constitutes evidence that the induced binding activities have specificity for DNA conformation, suggesting that they may also have functional significance, perhaps in DNA repair.

The induction of the DNA-binding activity was found to be UV dose-dependent, and so the induction of the DNA-binding proteins can be attributed to the effect of UV light on the cells. This again suggests that there may be a functional relationship between the induced DNA-binding proteins and DNA damage and repair. It was found that the accumulation of the DNA-binding activity follows a time course that peaks at about 18 hr after irradiation. This time course for induction suggests that the sensing by the cells of the UV-induced damage and their response to it in the form of DNA-binding proteins may be cell cycle-related.

The pathway by which UV light induces the DNA-binding proteins in HeLa cells is not known. A major product of UV irradiation of cells is DNA damage, and so our current hypothesis is that the induction by UV is mediated by its effects on DNA, but this has yet to be established. While DNA damage *per se* may be sufficient for the induction, it may not be necessary. The metabolic stress of serum starvation seems to be capable of inducing the same binding activity. It should be noted in this regard that Schorpp *et al.* (7) found that variations in the growth conditions of primary fibroblasts, such as serum starvation, could cause a high background level of induction of the PMA- and UV-inducible proteins they were studying. Since both UV irradiation and serum starvation of HeLa cells can inhibit DNA replication, it may be that it is the inhibition of DNA replication that is the common pathway in the induction of the DNA-binding activity we detect. In *E. coli*, inhibition of DNA replication is sufficient to induce at least some aspects of the SOS response (1).

The pathway of signal transduction from UV irradiation to increased DNA-binding activity is not known. The pathway may involve exclusively intracellular signals, or it may include extracellular factors in an autocrine manner. Initial attempts to detect an inducing factor in conditioned medium from UV-treated cells were not conclusive.

The induction of the DNA-binding proteins by UV light does not require *de novo* protein synthesis, and so it most likely involves modification of preexisting proteins either by phosphorylation or by proteolytic cleavage. This is not unprecedented, since the response of *E. coli* to UV involves the induction of new activities by protein modification (1). In a speculative model, one might imagine that different cellular stresses, such as DNA damage from UV light or growth arrest from serum starvation, may stimulate distinct but convergent pathways within the cell, leading to the activation of protein kinases or of specific proteases, which in turn act on preexisting proteins to induce DNA-binding activity. One example of this general mechanism in mammalian cells consists of the various pathways involving protein kinase C (13). Indeed, it may be that protein kinase C plays a role in the UV induction of DNA-binding proteins that we have observed, perhaps by phosphorylating and thereby activating the DNA-binding proteins. Another type of posttranslational modification that may be involved in the modulation of the DNA-binding activity is ADP-ribosylation of proteins. There is accumulating evidence that ADP-ribosylation may have a role in the regulation of DNA excision repair in human cells (14).

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